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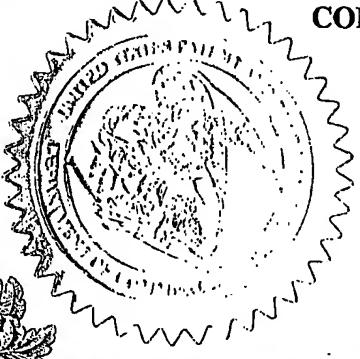
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# PROVISIONAL APPLICATION FOR PATENT COVER SHEET

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This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(b)(2).

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Additional inventors are being named on 1 separately numbered sheets attached hereto.

## TITLE OF THE INVENTION (280 characters max)

TUBERCULOSIS VACCINE WITH IMPROVED EFFICACY

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## ENCLOSED APPLICATION PARTS (check all that apply)

- |  |  |
|--|--|
| <input type="checkbox"/> Specification      Number of Pages [7]          | <input type="checkbox"/> CD(s), Number _____                                   |
| <input checked="" type="checkbox"/> Drawing(s)      Number of Sheets [1] | <input checked="" type="checkbox"/> Other (specify) Sequence Listing (5 pages) |
| <input type="checkbox"/> Application Data Sheet. See 37 CFR 1.76         |  |

## METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT (check one)

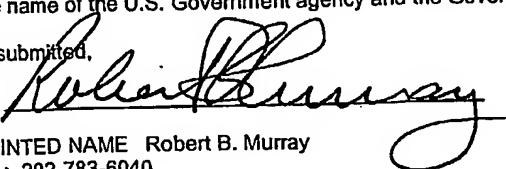
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|--|-----------------------------|
| <input type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27   | Filing Fee Amount: \$160.00 |
| <input type="checkbox"/> A check or money order is enclosed to cover the filing fee  |                             |
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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

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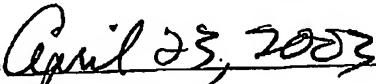
Yes, the name of the U.S. Government agency and the Government contract number are: \_\_\_\_\_

Respectfully submitted,

SIGNATURE 

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**USE ONLY FOR FILING PROVISIONAL APPLICATION FOR PATENT**

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Correspondence Customer Number:: 6449

**Application Information**

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**Representative Information**

Representative Customer Number:: 6449

- 1 -

## Tuberculosis Vaccine with Improved Efficacy

### Specification

5 The present invention relates to novel recombinant vaccines providing protective immunity especially against tuberculosis.

10 Tuberculosis (TB) caused by *Mycobacterium tuberculosis* remains a significant global problem. It is estimated that one third of the world's population is infected with *M.tuberculosis* (Kochl, 1991). In many countries the only measure for TB control has been vaccination with *M.bovis* bacille Calmette-Guérin (BCG). The overall vaccine efficacy of BCG against TB, however, is about 50 % with extreme variations ranging from 0 % to 80 % between different field trials (Roche et al., 1995). Thus, BCG should be improved, e.g. by genetic engineering, to provide a vaccine for better TB control (Murray et al., 1996; Hess and Kaufmann, 1993). The widespread emergence of multiple drug-resistant *M.tuberculosis* strains additionally underlines the urgent requirement for novel TB vaccines (Grange, 1996).

20 M.*tuberculosis* belongs to the group of intracellular bacteria that replicate within the phagosomal vacuoles of resting macrophages, thus protection against TB depends on T cell-mediated immunity (Kaufmann, 1993). Several studies in mice and humans, however, have shown that 25 Mycobacteria stimulate antigen-specific, major histocompatibility complex (MHC) class II- or class I-restricted CD4 and CD8 T cells, respectively (Kaufmann, 1993).

30 The important role of MHC class I-restricted CD8 T cells was convincingly demonstrated by the failure of  $\beta$ 2-microglobulin ( $\beta$ 2m) deficient mice to control experimental *M.tuberculosis* infection (Flynn et al., 1993). Because these mutant mice lack MHC class I, functional CD8 T cells cannot

- 2 -

develop. In contrast to *M.tuberculosis* infection,  $\beta$ 2m-deficient mice are capable of controlling certain infectious doses of the BCG vaccine strain (Flynn et al., 1993; Ladel et al., 1995). Furthermore, BCG vaccination of  $\beta$ 2m-deficient mice prolonged survival after subsequent *M.tuberculosis* infection whereas BCG-immunized C57BL/6 resisted TB (Flynn et al., 1993). This differential CD8 T cell dependency between *M.tuberculosis* and BCG may be explained as follows: *M.tuberculosis* antigens gain better access to the cytoplasm than antigens from BCG leading to more pronounced MHC class I presentation (Hess and Kaufmann, 1993). Consequently, a more effective CD8 T cell response is generated by *M.tuberculosis*. This notion was recently supported by increased MHC class I presentation of an irrelevant antigen, ovalbumin, by simultaneous *M.tuberculosis*, rather than BCG, infection of antigen presenting cells (APC) (Mazzaccaro et al., 1996).

15 Secreted proteins of *M.tuberculosis* comprise a valuable source of antigens for MHC class I presentation. Recently, a DNA vaccine encoding the secreted antigen Ag85A elicited MHC class I-restricted CD8 T cell responses in mice which may contribute to defence against TB (Huygen et al., 1996). In general, evidence is accumulating that immunization with secreted protein antigens of *M.tuberculosis* induce some protection against TB in guinea pigs and mice (Horwitz et al., 1995; Andersen, 1994). An important goal towards the development of improved TB vaccines based on BCG, therefore, is to augment the accessibility of secreted BCG-specific antigens to the cytoplasm of infected APC. Subsequent delivery of peptides derived from these secreted proteins into the MHC class I presentation pathway may potentiate the already existing BCG-specific immune response for preventing TB.

20 30 The phagolysosomal escape of *L.monocytogenes* represents a unique mechanism to facilitate MHC class I antigen presentation of listerial antigens (Berche et al., 1987; Portnoy et al., 1988). Listeriolysin (Hly), a

- 3 -

pore-forming sulphydryl-activated cytolsin, is essential for the release of *L.monocytogenes* microorganisms from phagolysosomal vacuoles into the cytosol of host cells (Gaillard et al., 1987; Portnoy et al., 1988). This escape function was recently transferred to *Bacillus subtilis* and to attenuated *Salmonella* ssp. strains (Bielecki et al., 1991; Gentschew et al., 1995; Hess and Kaufmann, 1997). Hly expression by an asporogenic *B.subtilis* mutant strain or in *Salmonella* ssp. results in bacterial escape from the phagolysosome into the cytosol of J774 macrophage-like cells (Bielecki et al., 1991; Gentschew et al., 1995; Hess and Kaufmann, 1997).

WO 99/101496 and Hess et al. (1998) disclose recombinant *Mycobacterium bovis* strains that secrete biologically active Listeriolysin fusion proteins. These *M.bovis* strains have been shown to be effective vaccines against TB in several animal models.

According to the present invention Hly was expressed in urease-deficient BCG strains. These urease-deficient BCG strains exhibit an increased Hly activity in phagosomes and in turn improved pore formation in the endosomal membranes leading to superior immunoprotectivity.

Thus, a first aspect of the present invention is a bacterial cell, particularly a *Mycobacterium* cell which is urease-deficient and comprises a recombinant nucleic acid molecule encoding a fusion polypeptide comprising (a) at least one domain from a polypeptide, wherein said polypeptide domain is capable of eliciting an immune response in a mammal, and (b) a phagolysosomal escape domain. It is preferred that the cell is capable of expressing the nucleic acid molecule of the invention. More preferably, the cell is capable of secreting the fusion polypeptide and/or of providing it in a form suitable for MHC class I-restricted antigen recognition.

- 4 -

The bacterial cell of the invention is a urease-deficient cell, e.g. a gram-negative or a gram-positive bacterial cell, preferably a Mycobacterium cell. The urease-deficiency may be achieved by partially or completely inactivating one or several cellular nucleic acid molecules which code for a urease subunit, particularly ureA encoding for urease subunit A, ureB coding for urease subunit B and/or ureC coding for urease subunit C. The sequences of ureA, ureB and ureC in Mycobacteria, particularly M.bovis and M.tuberculosis and the proteins encoded thereby are described by Reyrat et al. (1995) and Clemens et al. (1995), which are incorporated herein by reference.

Preferably the urease-deficient bacterial strain is obtained by deletions and/or insertions of one or several nucleotides in urease subunit - coding nucleic acid sequences and/or their expression control sequences. Deletions and/or insertions may be generated by homologous recombination, transposon insertion or other suitable methods.

In an especially preferred embodiment the ureC sequence is inactivated, e.g. by constructing a suicide vector containing a ureC gene disrupted by a selection marker gene, transforming the target cell with the vector and screening for selection marker-positive cells having a urease negative phenotype as described by Reyrat et al. (1995).

The cell of the invention is preferably an M.bovis cell, a M.tuberculosis cell, particularly an attenuated M.tuberculosis cell or other Mycobacteria, e.g. M.microti, M.smegmatis, M.canettii, M.marinum or M.fortuitum or Mycobacteria as described by Reyrat et al. (1995).

The Mycobacterium cell of the invention comprises a recombinant nucleic acid molecule, e.g. the nucleic acid molecule in SEQ ID No.1. This nucleic acid molecule comprises a signal peptide coding sequence (nucleotide 1 - 120), a sequence coding for an immunogenic domain (nucleotide 121 -

153), a peptide linker coding sequence (nucleotide 154 - 210), a sequence coding for a phagolysosomal domain (nucleotide 211 - 1722), a further peptide linker coding sequence (nucleotide 1723 - 1800) and a sequence coding for a random peptide (nucleotide 1801 - 1870). The corresponding amino acid sequence is shown in SEQ ID No.2.

The nucleic acid contains at least one immunogenic domain from a polypeptide. The immunogenic domain may be derived from an organism of the genus *Mycobacterium*, preferably from *Mycobacterium tuberculosis* or 10 from *Mycobacterium bovis*. This domain has a length of at least 6, preferably of at least 8 amino acids. The immunogenic domain is preferably a portion of a native *Mycobacterium* polypeptide. However, within the scope of the present invention is also a modified immunogenic domain, which is derived from a native immunogenic domain by substituting, 15 deleting and/or adding one or several amino acids.

The immunogenic domain is however not restricted to *Mycobacterium* antigens and can be selected from autoantigens, tumor antigens and pathogen antigens such as virus antigens, parasite antigens, bacterial 20 antigens in general and immunogenic fragments thereof. Specific examples for suitable tumor antigens are human tumor antigens such as the p53 tumor suppressor gene product (Houbiers et al., 1993) and melanocyte differentiation antigens, e.g. Melan-A/MART-1 and gp100 (van Elsas et al., 1996). Specific examples for suitable virus antigens are human tumor virus 25 antigens such as human papilloma virus antigens, e.g. antigens E6 and E7 (Bosch et al., 1991), influenza virus antigens, e.g. influenza virus nucleoprotein (Matsui et al., 1995; Fu et al., 1997) or retroviral antigens such as HIV antigens, e.g. the HIV-1 antigens p17, p24, RT and Env (Harrer et al., 1996; Haas et al., 1996). Specific examples for suitable 30 parasite antigens are *Plasmodium* antigens such as liver stage antigen (LSA-1), circumsporozoite protein (CS or allelic variants cp26 or cp29), thrombospondin related anonymous protein (TRAP), sporozoite threonine

- 6 -

and asparagine rich protein (STARP) from *Plasmodium falciparum* (Aidoo et al., 1995) and *Toxoplasma* antigens such as p30 from *Toxoplasma gondii* (Khan et al., 1991; Bulow and Boothroyd, 1991). Specific examples for suitable bacterial antigens are *Legionella* antigens such as Major secretary protein from *Legionella pneumophila* (Blander and Horwitz, 1991).

The immunogenic domain is capable of eliciting an immune response in a mammal. This immune response can be a B cell-mediated immune response. Preferably, however, the immunogenic domain is capable of eliciting a T cell-mediated immune response, more preferably a MHC class I-restricted CD8 T cell response.

The domain capable of eliciting an immune response is more preferably selected from immunogenic peptides or polypeptides from *M.bovis* or *M.tuberculosis* or from immunogenic fragments thereof. Specific examples for suitable antigens are Ag85B (p30) from *M.tuberculosis* (Harth et al., 1996), Ag85B ( $\alpha$ -antigen) from *M.bovis* BCG (Matsuo et al., 1988), Ag85A from *M.tuberculosis* (Huygen et al., 1996) and ESAT-6 from *M.tuberculosis* (Sorensen et al., 1996, Harboe et al., 1996 and Andersen et al., 1995). More preferably, the immunogenic domain is derived from the antigen Ag85B. Most preferably, the immunogenic domain comprises the sequence from aa,41 to aa,51 in SEQ ID No.2.

The recombinant nucleic acid molecule according to the present invention further comprises a phagolysosomal escape domain, i.e. a polypeptide domain which provides for an escape of the fusion polypeptide from the phagolysosome into the cytosol of mammalian cells. Preferably, the phagolysosomal escape domain is a *Listeria* phagolysosomal escape domain, which is described in US 5,733,151, herein incorporated by reference. More preferably, the phagolysosomal escape domain is derived from the organism *L.monocytogenes*. Most preferably, the phagolysosomal domain is encoded by a nucleic acid molecule selected from: (a) a

nucleotide sequence comprising nucleotides 211 - 1722 as shown in SEQ ID No.1, (b) a nucleotide sequence which encodes for the same amino acid sequence as the sequence from (a), and (c) a nucleotide sequence hybridizing under stringent conditions with the sequence from (a) or (b).

5

Apart from the nucleotide sequence depicted in SEQ ID No.1 the present invention also comprises nucleic acid sequences hybridizing therewith. In the present invention the term "hybridization" is used as defined in Sambrook et al. (Molecular Cloning. A laboratory manual, Cold Spring Harbor Laboratory Press (1989), 1.101-1.104). In accordance with the present invention the term "hybridization" is used if a positive hybridization signal can still be observed after washing for one hour with 1 X SSC and 0.1 % SDS at 55°C, preferably at 62° C and more preferably at 68°C, particularly for 1 hour in 0.2 X SSC and 0.1 % SDS at 55°C, preferably at 62°C and more preferably at 68°C. A sequence hybridizing with a nucleotide sequence as per SEQ ID No.1 under such washing conditions is a phagolysosomal escape domain encoding nucleotide sequence preferred by the subject invention.

20

A nucleotide sequence encoding a phagolysosomal escape domain as described above may be directly obtained from a Listeria organism or from any recombinant source e.g. a recombinant E.coli cell containing the corresponding Listeria nucleic acid molecule or a variant thereof as described above.

25

Preferably, the recombinant nucleic acid molecule encoding for a fusion polypeptide contains a signal peptide encoding sequence. More preferably, the signal sequence is a signal sequence active in Mycobacteria, preferably in M.bovis, e.g. a native M.bovis signal sequence. A preferred example of a suitable signal sequence is the nucleotide sequence coding for the Ag85B signal peptide which is depicted in SEQ ID No.1 from nucleotide 1 to 120.

- 8 -

Further, it is preferred that a peptide linker be provided between the immunogenic domain and the phagolysosomal escape domain. Preferably, said peptide linker has a length of from 5 to 50 amino acids. More preferably, a sequence encoding a linker as shown in SEQ ID No.1 from nucleotide 154 to 210 or a sequence corresponding thereto as regards the degeneration of the genetic code.

The nucleic acid may be located on a recombinant vector. Preferably, the recombinant vector is a prokaryotic vector, i.e. a vector containing elements for replication or/and genomic integration in prokaryotic cells. Preferably, the recombinant vector carries the nucleic acid molecule of the present invention operatively linked with an expression control sequence. The expression control sequence is preferably an expression control sequence active in Mycobacteria, particularly in M.bovis. The vector can be an extrachromosomal vector or a vector suitable for integration into the chromosome. Examples of such vectors are known to the man skilled in the art and, for instance, given in Sambrook et al. supra.

In a further aspect of the present invention a urease-deficient bacterial cell e.g., a Mycobacterium cell, preferably an M.bovis cell is provided which comprises at least one nucleic acid molecule encoding a phagolysosomal escape peptide or polypeptide. Even if the phagolysosomal escape peptide or polypeptide is not fused with an antigen, a surprising improvement of the immunogenic properties is found.

The recombinant bacterial cell which is provided according to this further aspect of the present invention may contain at least one further recombinant, e.g. heterologous nucleic acid molecule encoding a peptide or polypeptide capable of eliciting an immune response in a mammal. Said further immunogenic peptide or polypeptide may be selected from Mycobacterium antigens or, in a wider sense, from autoantigens, tumor antigens, pathogen antigens and immunogenic fragments thereof. The

nucleic acid molecule coding for the further peptide or polypeptide may be situated on the same vector as the fusion gene. However, it may, for example, also be situated on a different plasmid, independently of the fusion gene, or be chromosomally integrated.

5

Surprisingly, it was found that a *Mycobacterium* cell according to the present invention has an intracellular persistence in infected cells, e.g. macrophages, which is equal or less than the intracellular persistence of a corresponding native *Mycobacterium* cell which does not contain the recombinant nucleic acid molecule.

10

The present invention also refers to a pharmaceutical composition comprising as an active agent a cell as defined above, optionally together with pharmaceutically acceptable diluents, carriers and adjuvants.

15

Preferably, the composition is a living vaccine suitable for administration to a mammal, preferably a human. The actually chosen vaccination route depends on the choice of the vaccination vector. Administration may be achieved in a single dose or repeated at intervals. The appropriate dosage depends on various parameters such as the vaccinal vector itself or the route of administration. Administration to a mucosal surface (e.g. ocular, intranasal, oral, gastric, intestinal, rectal, vaginal or urinary tract) or via the parenteral route (e.g. subcutaneous, intradermal, intramuscular, intravenous or intraperitoneal) might be chosen.

20

Further, the present invention pertains to a method for preparing a recombinant bacterial cell as defined above. According to the first aspect, this method comprises the steps of (i) providing a urease-deficient bacterial cell, particularly a *Mycobacterium* cell, (ii) inserting a recombinant nucleic acid molecule into said bacterial cell, said nucleic acid molecule encoding a fusion polypeptide comprising (a) at least one domain from a polypeptide wherein said domain is capable of eliciting an immune response in a mammal and (b) a phagolysosomal escape domain, and (iii) cultivating the

- 10 -

cell obtained according to step (ii) under suitable conditions. Preferably, a cell is obtained which is capable of expressing said nucleic acid molecule. More preferably, the cell is an *M.bovis* cell.

5 According to the further aspect, this method comprises the step of (i) providing an urease-deficient bacterial cell, particularly a *Mycobacterium* cell, (ii) inserting a recombinant nucleic acid molecule into said bacterial cell, said nucleic acid molecule encoding a phagolysosomal escape peptide or polypeptide, and (iii) cultivating the cell obtained according to (ii) under  
10 suitable conditions.

If desired, the method of the present invention comprises inserting at least one further recombinant nucleic acid molecule into the bacterial cell, said further recombinant nucleic acid molecule encoding a peptide or polypeptide capable of eliciting an immune response in a mammal.  
15

Finally, the present invention relates to a method for the preparation of a living vaccine comprising formulating the recombinant cell in a pharmaceutically effective amount with pharmaceutically acceptable  
20 diluents, carriers and/or adjuvants.

The invention will be further illustrated by the following figures and sequence listings.

25 Fig.1: the protective capacity of r<sub>aureC</sub> BCG Hly in the aerosol model of murine tuberculosis. BALB/c mice were immunized i.v. with  $1 \times 10^8$  CFU r<sub>aureC</sub> BCG Hly or BCG "Pasteur". 120 days post vaccination animals were challenged with H37Rv (200 organism/lung) via aerosol. Bacterial load in infected  
30 organs (spleen and lung) was assessed 30, 60 and 90 days post challenge. Each bar represents 10 animals.

- 11 -

SEQ ID No.1: shows the nucleotide sequence of a nucleic acid molecule according to the present invention.

SEQ ID No.2: shows the corresponding amino acid sequence of the nucleic acid molecule of SEQ ID No.1.

Example

1. Inactivation of the urease activity of BCG delta ureC.

To obtain a urease-deficient mutant, Reyrat et al. constructed a suicide vector containing a ureC gene disrupted by a kanamycin marker (the aph gene). Two micrograms of this construct were linearized with Sac I and electroporated into *M. bovis* BCG. Kanamycine resistant transformants were screened for urease negative phenotype (cf. Reyrat et al., 1995).

2. Construction of the mycobacterial *E. coli* shuttle expression vector pMV306:Hly.

To transfer the phagosomal escape function (mediated by Hly of *L. monocytogenes* EGD Sv 1/2a), to BCG Pasteur (1173 P<sub>3</sub>) delta ureC, an *E. coli*-mycobacterial shuttle vector was used. The intergrative plasmid pMV306, a precursor of vector pMV361, allows stable chromosomal expression of Hly.

A pILH-1-derived 1.7-kb PstI DNA fragment coding for an hly-hlyA (*E. coli* pHly152-specific hemolysin A) ORF was inserted into PstI site of plasmid pAT261. This resulting gene fusion codes for the expression of secreted proteins directed to the supernatant by the BCG-specific Ag85B signal peptide. The construct was termed pAT261:Hly and its XbaI-Sall DNA expression cassette under

- 12 -

transcriptional control of the hsp60 mycobacterial promoter was subsequently used for insertion into the parental pMV306 vector resulting in the construct pMV306:Hly. The DNA sequence of the hly-specific insertion sites in both mycobacterial expression plasmids was analyzed. The mature Hly fusion protein putatively consists of 30 aa at the N terminus and 52 aa at the C-terminal part of the fusion that partially belong to HlyA of *E. coli*.

### 3. Protective capacity

The expression vector pMV306:Hly was transformed into an urease deficient BCG strain. The resultant strain was designated r ΔureC/BCG Hly. The protective capacity of this urease-deficient mycobacterial strain in a model of murine tuberculosis is shown in Figure 1.

15

## References

- Aidoo, M., Lalvani, A., Allsopp, C.E.M. et al. (1995), Identification of conserved antigenic components for a cytotoxic T lymphocyte-inducing vaccine against malaria, *The Lancet* 345: 1003.
- Andersen, P. (1994), Effective vaccination of mice against *Mycobacterium tuberculosis* infection with a soluble mixture of secreted *Mycobacterial* protein, *Infect. Immun.* 62: 2536-2544.
- 10 Andersen, P., Andersen, A.B., Sorensen, A.L. and Nagai, S. (1995), Recall of long-lived immunity to *Mycobacterium tuberculosis* infection in mice, *J. Immunol.* 154: 3359.
- 15 Berche, P., Gaillard, J.L., and Sansonetti, P.J. (1987), Intracellular growth of *L.monocytogenes* as a prerequisite for in vivo induction of T cell-mediated immunity, *J. Immunol.* 138: 2266-2276.
- Bielecki, J., Youngman, P., Connelly, P., and Portnoy, D.A... (1990),  
20 *Bacillus subtilis* expressing a hemolysin gene from *Listeria monocytogenes* can grow in mammalian cells, *Nature* 354: 175-176.
- Blander, S.J. and Horwitz, M.A. (1991), Vaccination with a major secretory protein of *Legionella* induces humoral and cell-mediated immune  
25 responses and protective immunity across different serogroups of *Legionella pneumophila* and different species of *Legionella*, *J. Immunol.* 147: 285.
- Bosch, F.X., Durst, M., Schwarz, E., Boukamp, P., Fusenig, N.E. and zur  
30 Hausen, H. (1991), The early genes E6 and E7 of cancer associated human papilloma viruses as targets of tumor suppression?, *Behring Inst. Mitt.* 108.

- 14.

- Bulow, R. and Boothroyd, J.C. (1991), Protection of mice from fatal *Toxoplasma gondii* infection by immunization with p30 antigen in liposomes, *J. Immunol.* 147: 3496.
- 5 Clemens, D.L., and Horwitz, M.A. (1996), The *Mycobacterium tuberculosis* phagosome interacts with early endosomes and is accessible to exogenously administered transferrin, *J. Exp. Med.* 184: 1349-1355.
- 10 Clemens, D.L., Lee B.Y., Horwitz, M.A. (1995), Purification, characterization, and genetic analysis of *Mycobacterium tuberculosis* urease, a potentially critical determinant of host-pathogen interaction. *J. Bacteriol.* 1995 177:5644-5652.
- 15 Darji, A., Chakraborty, T., Weiland, J., and Weiss, S. (1996), Listeriolysin generates a route for the presentation of exogenous antigens by major histocompatibility complex class I, *Eur. J. Immunol.* 25: 2967-2971.
- 20 Domann, E., and Chakraborty, T. (1989), Nucleotide sequence of the listeriolysin gene from a *Listeria monocytogenes* serotype 1 / 2a strain, *Nucleic Acids Res.* 17: 6406.
- 25 Flesch, I., Hess, J.H., Oswald, I.P., and Kaufmann, S.H.E. (1994), Growth inhibition of *Mycobacterium bovis* by IFN- $\gamma$  stimulated macrophages: regulation by endogenous tumor necrosis factor- $\alpha$  and by IL-10, *Int. Immunol.* 6: 693-700.
- 30 Flynn, J.L., Goldstein, M.M., Triebold, K.J., Koller, B., and Bloom, B.R. (1992), Major histocompatibility complex class I-restricted T cells are required for resistance to *Mycobacterium tuberculosis* infection, *Proc. Natl. Acad. Sci. USA* 89: 12013-12017.

- 15 -

Fu, T.M., Friedman, A., Ulmer, J.B., Liu, M.A. and Donnelly, J.J. (1997), Protective cellular immunity: cytotoxic T-lymphocyte responses against dominant and recessive epitopes of influenza virus nucleoprotein induced DNA immunization, *J. Virol.* 71: 2715.

5 Gaillard, J.L., Berche, P., Mounier, J., Richard, S., and Sansonetti, P.J. (1987), In vitro model of penetration and intracellular growth of *Listeria monocytogenes* in the human enterocyte-like cell line Caco-2, *Infect. Immun.* 55: 2822-2829.

10 Gentschew, I., Sokolovic, Z., Mollenkopf, H.-J., Hess, J., Kaufmann, S.H.E., Kuhn, M., Krohne, G.F., and Goebel, W. (1995), *Salmonella* secreting active listeriolysin changes its intracellular localization, *Infect. Immun.* 63: 4202-4205.

15 Grange, J.M. (1996), Epidemiological aspects of drug resistance, in *Mycobacteria and human disease*, Arnold, London, pp. 124-125.

20 Haas, G., Plikat, U., Debre, P., Lucchiari, M., Katlama, C., Dudoit, Y., Bonduelle, O., Bauer, M., Ihlenfeldt, H.G., Jung, G., Maier, B., Meyerhans, A. and Autran, B. (1996), Dynamics of viral variants in HIV-1 Nef and specific cytotoxic T lymphocytes in vivo, *J. Immunol.* 157: 4212.

25 Harboe, M., Oettinger, T., Wiker, H.G. et al. (1996), Evidence for occurrence of the ESAT-6 protein in *Mycobacterium tuberculosis* and virulent *Mycobacterium bovis* and for its absence in *Mycobacterium bovis* BCG, *Infect. Immun.* 64: 16.

- 16 -

- Harrer, T., Harrer, E., Kalams, S.A., Barbosa, P., Trocha, A., Johnson, R.P., Elbeik, T., Feinberg, M.B., Buchbinder, S.P. and Walker, B.D. (1996), Cytotoxic T lymphocytes in asymptomatic long-term nonprogressing HIV-1 infection. Breadth and specificity of the response and relation to in vivo viral quasispecies in a person with prolonged infection and low viral load, J. Immunol. 156: 2616.
- Harth, G., Lee, B.-Y., Wang, J., Clemens, D.L., and Horwitz, M.A. (1996), Novel insights into the genetics, biochemistry, and immunocytochemistry of the 30-kilodalton major extracellular protein of *Mycobacterium tuberculosis*, Infect. Immun. 64: 3038-3047.
- Hess, J., Weis, W., Vogel, M., and Goebel, W. (1986), Nucleotide sequence of plasmid-encoded hemolysin determinant and its comparison with a corresponding chromosomal hemolysin sequence, FEMS Lett. 34: 1-11.
- Hess, J., and Kaufmann, S.H.E. (1993), Vaccination strategies against intracellular microbes, FEMS Microbiol. Immunol. 7: 95-103.
- Hess, J., Gentschev, I., Miko, D., Welzel, M., Ladel, C., Goebel, W., and Kaufmann, S.H.E. (1996), Superior efficacy of secreted over somatic p60 or listeriolysin antigen display in recombinant *Salmonella* vaccine induced protection against listeriosis, Proc. Natl. Acad. Sci. USA 93: 1458-1463.
- Hess, J., and Kaufmann, S.H.E. (1997), Principles of cell-mediated immunity underlying vaccination strategies against intracellular pathogens, in Host Response to Intracellular Pathogens, S.H.E. Kaufmann (ed), R.G. Landes Co., Austin, pp. 75-90.

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- 17 -

Hess J., Miko D., Catic A., Lehmensiek V., Russell DG., Kaufmann SH., (1998), Mycobacterium bovis Bacille Calmette-Guerin strains secreting listeriolysin of Listeria monocytogenes. Proc Natl Acad Sci USA. 95(9):5299-304.

Horwitz, M.A., Lee, B.-W. E., Dillon, B.J., and Harth, G. (1995), Protective immunity against tuberculosis induced by vaccination with major extracellular proteins of Mycobacterium tuberculosis, Proc. Natl. Acad. Sci. USA 92: 1530-1534.

Houbiers, J.G.A., Nijman, H.W., van der Burg, S.H., Drijfhout, J.W., Kenemans, P., van de Velde, C.J.H., Brand, A., Momburg, F., Kast, W.M. and Melief, C.J.M. (1993), In vitro induction of human cytotoxic T lymphocyte responses against peptides of mutant and wild-type p53, Eur. J. Immunol. 23: 2072.

Huygen, K., Content, J., Denis, O., Montgomery, D.L., Yawman, A.M., Deck, R.R., DeWitt, C.M., Orme, I.M., Baldwin, S., D'Souza, C., Drowart, A., Lozes, E., Vandenbussche, P., Van Vooren, J.-P., Liu, M.A., and Ulmer, J.B. (1996), Immunogenicity and protective efficacy of a tuberculosis DNA vaccine, Nat. Med. 2: 893-898.

Kaufmann, S.H.E. (1993), Immunity to intracellular bacteria, Annu. Rev. Immunol. 11: 129-163.

Khan, I.A., Ely, K.H. and Kasper, L.H. (1991), A purified parasite antigen (p30) mediates CD8 T cell immunity against fatal Toxoplasma gondii infection in mice, J. Immunol. 147: 3501.

- 18 -

- King, C.H., Mundayoor, S., Crawford, J.T. and Shinnik, T.M. (1993), Expression of contact-dependent cytolytic activity by *Mycobacterium tuberculosis* and isolation of the genomic locus that encodes the activity, *Infect. Immun.* 61: 2708-2712.
- Kochi, A. (1991), The global tuberculosis situation and the new control strategy of the World Health Organization, *Tubercle* 72: 1-6.
- Ladel, C.H., Daugelat, S., and Kaufmann, S.H.E. (1995), Immune response to *Mycobacterium bovis* bacille Calmette Guérin Infection in major histocompatibility complex class I- and II-deficient knock-out mice: contribution of CD4 and CD8 T cells to acquired resistance, *Eur. J. Immunol.* 25: 377-384.
- Laemmli, U.K. (1970), Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature* 227: 680-685.
- Langermann, S., Palaszynski, S.R., Burlein, J.E., Koenig, S., Hanson, M.S., Briles, D.E., and Stover, C.K. (1994), Protective humoral response against pneumococcal infection in mice elicited by recombinant Bacille Calmette-Guérin vaccines expressing pneumococcal surface protein A., *J. Exp. Med.* 180: 2277-2286.
- Matsui, M., Moots, R.J., Warburton, R.J., Peace-Brewer, A., Tussey, L.G., Quinn, D.G., McMichael, A.J. and J.A. Frelinger (1995), Genetic evidence for differences between intracellular peptides of influenza A matrix peptide-specific CTL recognition, *J. Immunol.* 154: 1088.
- Matsuo, K., Yamaguchi, R., Yamazaki, A., Tasaka, H., Terasaka, K., and Yamada, T. (1990), Cloning and expression of the *Mycobacterium bovis* BCG gene for extracellular alpha antigen, *J. Bacteriol.* 170: 3847-3854.

- 19 -

- Mazzaccaro, R.Z., Gedde, M., Jensen, E.R., Van Santen, H.M., Ploegh  
H.L., Rock, K.L., and Bloom, B.R. (1996), Major histocompatibility class I  
presentation of soluble antigen facilitated by *Mycobacterium tuberculosis*  
infection, Proc. Natl. Acad. Sci. USA 93: 11786-11791.
- 5  
McDonough, K.A., Kress, Y., and Bloom, B.R. (1993), Pathogenesis of  
tuberculosis: Interaction of *Mycobacterium tuberculosis* with macrophages,  
Infect. Immun. 61: 2763-2773.
- 10 Murray, P.J., Aldovini, A., and Young, R.A. (1996), Manipulation and  
potentiation of anti-mycobacterial immunity using recombinant bacille  
Calmette-Guérin strains that secrete cytokines, Proc. Natl. Acad. Sci. USA  
93: 934-939.
- 15 Nato, F., Reich, K., Lhopital, S., Rouye, S., Geoffroy, C., Mazie, J.C.; and  
Cossart, P. (1991), Production and characterization of neutralizing and  
non-neutralizing monoclonal antibodies against listeriolysin O., Infect.  
Immun. 59: 4641-4646.
- 20 Portnoy, D.A., Jacks, P.S., and Hinrichs, D.J. (1988), Role of hemolysin  
for the intracellular growth of *Listeria monocytogenes*, J. Exp. Med. 167:  
1459-1471.
- 25 Reyrat J.M., Berthet F.X., Gicquel B., (1995) The urease locus of  
*Mycobacterium tuberculosis* and its utilization for the demonstration of  
allelic exchange in *Mycobacterium bovis* bacillus Calmette-Guerin. Proc  
Natl Acad Sci USA. 92(19):8768-72.
- Roche, P.W., Triccas, J.A., and Winter, N. (1995), BCG vaccination  
30 against tuberculosis: past disappointments and future hopes, Trends  
Microbiol. 3: 397-401.

- 20 -

- Russell, D.G. (1995), *Mycobacterium and Leishmania: stowaways in the endosomal network*. Trends in Cell Biology 5: 125-128.
- 5 Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989), *Molecular cloning: a laboratory manual*, 2nd edition, Cold Spring Harbor Laboratory Press, New York.
- 10 Schoel, B., Welzel, M., and Kaufmann, S.H.E. (1994), *Hydrophobic interaction chromatography for the purification of cytolytic bacterial toxins*, J. Chromatography A 667: 131-139.
- 15 Sorensen, A.L., Nagai, S., Houen, G., Andersen, P. and Andersen, A.B. (1995), *Purification and characterization of a low-molecular-mass-T-cell antigen secreted by Mycobacterium tuberculosis*, Infect. Immun. 63: 1710.
- 20 Stover, C.K., Bansal, G.P., Hanson, M.S., Burlein, J.E., Palaszynski, S.R., Young, J.F., Koenig, S., Young, D.B., Sadziene, A., Barbour, A.G. (1993), *Protective immunity elicited by recombinant Bacille Calmette Guérin (BCG) expressing outer surface protein A (OspA) lipoprotein: A candidate lyme disease vaccine*, J. Exp. Med. 178: 197-209.
- 25 Stover, C.K., de la Cruz, V.F., Fuerst, T.R., Burlein, J.E., Benson, L.A., Bennett, L.T., Bansal, G.P., Young, J.F., Lee, M.H., Hatfull, G.F., Snapper, S.B., Barletta, R.G., Jacobs, W.R., Jr., and Bloom, B.R. (1991), *New use of BCG for recombinant vaccines*, Nature 351: 456-460.
- 30 Sturgill-Koszycki, S., Schlesinger, P.H., Chakraborty, P., Haddix, P.L., Collins, H.L., Fok, A.K., Allen, R.D., Gluck, S.L., Heuser, J. and Russell, D.G. (1994), *Lack of acidification in Mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase*, Science 263: 678-681.

- 21 -

- Towbin, H., Staehelin, T., and Gordon, J. (1979), Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications, Proc. Natl. Acad. Sci. USA 76: 4350-4354.
- 5 Tsuchiya, S., Kobayashi, Y., Goto, Y., Okumura, H., Nakae, S., Konno, T., and Tada, K. (1982), Induction of maturation in cultured human monocytic leukemia cells by a phorbol diester, Cancer Res. 42: 1530-1536.
- 10 Tweten, R.K. (1995), Pore-forming toxins of gram-positive bacteria, in Virulence Mechanisms of Bacterial Pathogens, J.A. Roth et al. (ed), American Society for Microbiology, Washington, D.C., pp. 207-228.
- 15 van Elsas, A., van der Burg, S.H., van der Minne, C.E., Borghi, M., Mourer, J.S., Melief, C.J.M. and Schrier, P.I. (1996), Peptide-pulsed dendritic cells induce tumorcidal cytotoxic T lymphocytes from healthy donors against stably HLA-A\*0201-binding peptides from Melan-A/MART-1 self antigen, Eur. J. Immunol. 26: 1683.

## Claims

1. A bacterial cell which is urease-deficient and which comprises a recombinant nucleic acid molecule encoding a fusion polypeptide comprising (a) at least one domain from a polypeptide, wherein said polypeptide domain is capable of eliciting an immune response in a mammal, and (b) a phagolysosomal escape domain.
- 10 2. The cell of claim 1 wherein at least one cellular urease subunit encoding nucleic acid sequence is inactivated.
- 15 3. The cell of claim 2 wherein at least the cellular urease C subunit-encoding sequence is inactivated.
4. The cell of claim 1, wherein said phagolysosomal escape domain is a Listeria phagolysosomal escape domain.
- 20 5. The cell of claim 1, wherein said phagolysosomal domain is encoded by a nucleic acid molecule selected from:
  - (a) a nucleotide sequence comprising nucleotide 211 - 1722 as shown in SEQ ID No.1;
  - (b) a nucleotide sequence which encodes for the same amino acid sequence as the sequence from (a), and
  - 25 (c) a nucleotide sequence hybridizing under stringent conditions with the sequence from (a) or (b).
- 30 6. The cell of claim 1, wherein the domain capable of eliciting an immune response is a peptide or polypeptide capable of eliciting MHC class I-restricted CD8 T cell responses.

- 23 -

7. The cell of claim 1 wherein the domain capable of eliciting an immune response is from a Mycobacterium polypeptide.
8. The cell of claim 7, wherein the domain capable of eliciting an immune response is selected from the Mycobacterium antigens Ag85B (M.tuberculosis), Ag85B (M.bovis), Ag85A (M.tuberculosis) and ESAT-6 (M.tuberculosis) or an immunogenic fragment thereof.
9. The cell of claim 8, wherein the domain capable of eliciting an immune response is the antigen Ag85B or an immunogenic fragment thereof.
10. The cell of claim 1, wherein the fusion polypeptide is preceded by a signal peptide sequence.
11. The cell of claim 1, wherein a peptide linker is located between the immune response eliciting domain and the phagolysosomal domain.
12. The cell of claim 1, wherein said nucleic acid molecule is operatively linked with an expression control sequence.
13. The cell of claim 12, wherein said expression control sequence is active in said cell.
14. The cell of claim 1, wherein said nucleic acid molecule is located on a vector.
15. The cell of claim 1 which is a Mycobacterium cell.
16. The cell of claim 16 which is a Mycobacterium bovis cell.

- 24 -

17. A bacterial cell which is urease-deficient and which comprises at least one recombinant nucleic acid molecule encoding a phagolysosomal escape peptide or polypeptide.

18. The cell of claim 17, which comprises at least one further recombinant nucleic acid molecule encoding a peptide or polypeptide capable of eliciting an immune response in a mammal.

19. The cell of claim 18 which is a *Mycobacterium* cell.

20. The cell of claim 19 which is a *Mycobacterium bovis* cell.

21. The cell of claims 1 or 17, wherein the domain or peptide or polypeptide capable of eliciting an immune response is selected from autoantigens, tumor antigens, virus antigens, parasite antigens, bacterial antigens and immunogenic fragments thereof.

22. The cell of claims 1 or 17, which is capable of expressing said at least one recombinant nucleic acid molecule.

23. The cell of claim 22, which is capable of secreting a polypeptide encoded by said at least one nucleic acid molecule.

24. The cell of claims 1 or 23, which has an intracellular persistence in infected macrophages which is equal or less than the intracellular persistence of a native *Mycobacterium* cell.

25. A pharmaceutical composition comprising as an active agent a cell of claims 1 or 17, optionally together with pharmaceutically acceptable diluents, carriers and adjuvants.

- 26 -

26. The composition of claim 25, which is a living vaccine suitable for administration to a mucosal surface or via the parenteral route.

27. A method for the preparation of a living vaccine comprising formulating a cell of claims 1 or 17 in a pharmaceutically effective amount with pharmaceutically acceptable diluents, carriers and adjuvants.

28. A method for preparing a recombinant bacterial cell of claim 1 comprising the steps:

- (i) providing a urease-deficient bacterial cell;
- (ii) inserting a recombinant nucleic acid molecule into said bacterial cell, said nucleic acid molecule encoding a fusion polypeptide comprising (a) at least one domain from a polypeptide, wherein said domain is capable of eliciting an immune response in a mammal, and (b) a phagolysosomal escape domain, and
- (iii) cultivating the cell obtained according to (ii) under suitable conditions.

29. The method of claim 28, wherein said cell is a *M.bovis* cell.

30. A method for preparing a recombinant bacterial cell of claim 17 comprising the steps:

- (i) providing a urease-deficient bacterial cell;
- (ii) inserting a recombinant nucleic acid molecule into said bacterial cell, said nucleic acid molecule encoding a phagolysosomal escape peptide or polypeptide and
- (iii) cultivating the cell obtained according to (ii) under suitable conditions.

- 26 -

31. The method of claim 30 comprising inserting at least one further recombinant nucleic acid molecule into the bacterial cell, said further recombinant nucleic acid molecule encoding a peptide or polypeptide capable of eliciting an immune response in a mammal.
- 5
32. The method of claim 28 or 30, wherein the domain or peptide or polypeptide capable of eliciting an immune response is selected from autoantigens, tumor antigens, virus antigens, parasite antigens, bacterial antigens and immunogenic fragments thereof.

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-27-

**Abstract**

The present invention relates to novel recombinant vaccines providing  
5 protective immunity against tuberculosis. Further, the present invention  
refers to novel recombinant nucleic acid molecules, vectors containing said  
nucleic acid molecules, cells transformed with said nucleic acid molecules  
and polypeptides encoded by said nucleic acid molecules.

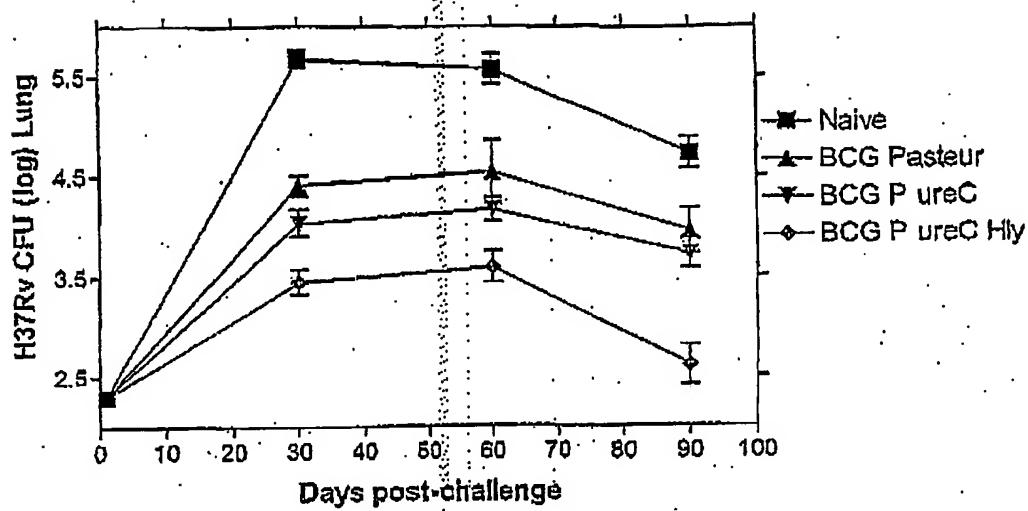
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15 kt/ANM/29625P US-22.04.03

Attorney Dkt # 2004-0644-002503  
Brode, et al.

-1/1-

Figure 1



## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- 5                   (i) APPLICANT:  
                      (A) NAME: Max-Planck-Gesellschaft zur Foerderung  
                       der  
                       Wissenschaften e.V.  
 10                 (B) STREET: Hofgartenstrasse 2  
                      (C) CITY: Muenchen  
                      (E) COUNTRY: Germany  
                      (F) POSTAL CODE (ZIP): 80539
- 15                 (ii) TITLE OF INVENTION: Tuberculosis vaccine
- 15                 (iii) NUMBER OF SEQUENCES: 2
- 20                 (iv) COMPUTER READABLE FORM:  
                      (A) MEDIUM TYPE: Floppy disk  
                      (B) COMPUTER: IBM PC compatible  
                      (C) OPERATING SYSTEM: PC-DOS/MS-DOS  
                      (D) SOFTWARE: PatentIn Release #1.0, Version  
                      #1.30 (EPO)

## 25                 (2) INFORMATION FOR SEQ ID NO: 1:

- 30                 (i) SEQUENCE CHARACTERISTICS:  
                      (A) LENGTH: 1881 base pairs  
                      (B) TYPE: nucleic acid  
                      (C) STRANDEDNESS: both  
                      (D) TOPOLOGY: linear

- 35                 (ix) FEATURE:  
                      (A) NAME/KEY: CDS  
                      (B) LOCATION: 1..1878

## 35                 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

40	ATG ACA GAC GTG AGC CGA AAG ATT CGA GCT TGG GGA CGC CGA TTG ATG Met Thr Asp Val Ser Arg Lys Ile Arg Ala Trp Gly Arg Arg Leu Met 1                       5                       10                       15	48
45	ATC GGC ACG GCA GCG GCT GTA GTC CTT CCG GGC CTG GTG GGG CTT GCC Ile Gly Thr Ala Ala Ala Val Val Leu Pro Gly Leu Val Gly Leu Ala 20                       25                       30	96
50	GGC GGA GCG GCA ACC GCG GGC GCG TTC TCC CCG CCG GGG CTG CCG GTC Gly Gly Ala Ala Thr Ala Gly Ala Phe Ser Arg Pro Gly Leu Pro Val 35                       40                       45	144
55	GAG TAC CTG CAG TCT GCA AAG CAA TCC GCT GCA AAT AAA TTG CAC TCA Glu Tyr Leu Gln Ser Ala Lys Gln Ser Ala Ala Asn Lys Leu His Ser 50                       55                       60	192
60	GCA GGA CAA AGC ACG AAA GAT GCA TCT GCA TTC AAT AAA GAA AAT TCA Ala Gly Gln Ser Thr Lys Asp Ala Ser Ala Phe Asn Lys Glu Asn Ser 65                       70                       75                       80	240
65	ATT TCA TCC ATG GCA CCA CCA GCA TCT CCG CCT GCA AGT CCT AAG ACG Ile Ser Ser Met Ala Pro Pro Ala Ser Pro Pro Ala Ser Pro Lys Thr 85                       90                       95	288

	CCA ATC GAA AAG AAA CAC GCG GAT GAA ATC GAT AAG TAT ATA CAA GGA Pro Ile Glu Lys Lys His Ala Asp Glu Ile Asp Lys Tyr Ile Gln Gly 100	105	110	336
5	TTG GAT TAC AAT AAA AAC AAT GTA TTA GTA TAC CAC GGA GAT GCA GTG Leu Asp Tyr Asn Lys Asn Asn Val Leu Val Tyr His Gly Asp Ala Val 115	120	125	384
10	ACA AAT GTG CCG CCA AGA AAA GGT TAC AAA GAT GGA AAT GAA TAT ATT Thr Asp Val Pro Pro Arg Lys Gly Tyr Lys Asp Gly Asn Glu Tyr Ile 130	135	140	432
15	GTT GTG GAG AAA AAG AAG AAA TCC ATC AAT CAA AAT AAT GCA GAC ATT Val Val Glu Lys Lys Ser Ile Asp Gln Asn Asn Ala Asp Ile 145	150	155	480
	CAA GTT GTG AAT GCA ATT TCG AGC CTA ACC TAT CCA GGT GCT CTC GTA Gln Val Val Asn Ala Ile Ser Ser Leu Thr Tyr Pro Gly Ala Leu Val 165	170	175	528
20	AAA GCG AAT TCG GAA TTA GTA GAA AAT CAA CCA GAT GTT CTC CCT GTA Lys Ala Asn Ser Glu Leu Val Glu Asn Gln Pro Asp Val Leu Pro Val 180	185	190	576
25	AAA CGT GAT TCA TTA ACA CTC AGC ATT GAT TTG CCA GGT ATG ACT AAT Lys Arg Asp Ser Leu Thr Leu Ser Ile Asp Leu Pro Gly Met Thr Asn 195	200	205	624
30	CAA GAC AAT AAA ATC GTT GTA AAA AAT GGC ACT AAA TCA AAC GTT AAC Gln Asp Asn Lys Ile Val Val Lys Asn Ala Thr Lys Ser Asn Val Asn 210	215	220	672
	AAC GCA GTA AAT ACA TTA GTG GAA AGA TGG AAT GAA AAA TAT GCT CAA Asn Ala Val Asn Thr Leu Val Glu Arg Trp Asn Glu Lys Tyr Ala Gln 225	230	235	720
35	GCT TAT CCA AAT GTA AGT GCA AAA ATT GAT TAT GAT GAC GAA ATG GCT Ala Tyr Pro Asn Val Ser Ala Lys Ile Asp Tyr Asp Asp Glu Met Ala 245	250	255	768
40	TAC AGT GAA TCA CAA TTA ATT GCG AAA TTT GGT ACA GCA TTT AAA GCT Tyr Ser Glu Ser Gln Leu Ile Ala Lys Phe Gly Thr Ala Phe Lys Ala 260	265	270	816
45	GTA AAT AAT AGC TTG AAT GTA AAC TTC GGC GCA ATC AGT GAA GGG AAA Val Asn Asn Ser Leu Asn Val Asp Phe Gly Ala Ile Ser Glu Gly Lys 275	280	285	864
50	ATG CAA GAA GAA GTC ATT AGT TTT AAA CAA ATT TAC TAT AAC GTG AAT Met Gln Glu Glu Val Ile Ser Phe Lys Gln Ile Tyr Tyr Asn Val Asn 290	295	300	912
	GTT AAT GAA CCT ACA AGA CCT TCC AGA TTT TTC GGC AAA GCT GTT ACT Val Asn Glu Pro Thr Arg Pro Ser Arg Phe Phe Gly Lys Ala Val Thr 305	310	315	960
55	AAA GAG CAG TTG CAA GCG CTT GGA GTG AAT GCA GAA AAT CCT CCT GCA Lys Glu Gln Leu Gln Ala Leu Gly Val Asn Ala Glu Asn Pro Pro Ala 325	330	335	1008
60	TAT ATC TCA AGT GTG GCG TAT GGC CGT CAA GTT TAT TTG AAA TTA TCA Tyr Ile Ser Ser Val Ala Tyr Gly Arg Gln Val Tyr Leu Lys Leu Ser 340	345	350	1056
65	ACT AAT TCC CAT AGT ACT AAA GTA AAA GCT GCT TTT GAT GCT GCC GTA Thr Asn Ser His Ser Thr Lys Val Lys Ala Ala Phe Asp Ala Ala Val 355	360	365	1104
70	AGC GGA AAA TCT GTC TCA GGT GAT GTA GAA CTA ACA AAT ATC ATC AAA Ser Gly Lys Ser Val Ser Gly Asp Val Glu Leu Thr Asn Ile Ile Lys 370	375	380	1152

	AAT TCT TCC TTC AAA GCC GTA ATT TAC GGA GGT TCC GCA AAA GAT GAA Asn Ser Ser Phe Lys Ala Val Ile Tyr Gly Gly Ser Ala Lys Asp Glu 385 390 395 400	1200
5	GTT CAA ATC ATC GAC GGC AAC CTC GGA GAC TTA CGC GAT ATT TTG AAA Val Gin Ile Ile Asp Gly Asn Leu Gly Asp Leu Arg Asp Ile Leu Lys 405 410 415	1248
10	AAA GGC GCT ACT TTT AAT CGA GAA ACA CCA GGA GTT CCC ATT GCT TAT Lys Gly Ala Thr Phe Asn Arg Glu Thr Pro Gly Val Pro Ile Ala Tyr 420 425 430	1296
15	ACA ACA AAC TTC CTA AAA GAC AAT GAA TTA CCT GTT ATT AAA AAC AAC Thr Thr Asn Phe Leu Lys Asp Asn Glu Leu Ala Val Ile Lys Asn Asn 435 440 445	1344
20	TCA GAA TAT ATT GAA ACA ACT TCA AAA CCT TAT ACA GAT GGA AAA ATT Ser Glu Tyr Ile Glu Thr Thr Ser Lys Ala Tyr Thr Asp Gly Lys Ile 450 455 460	1392
25	AAC ATC GAT CAC TCT GGA GGA TAC GTT GCT CAA TTC AAC ATT TCT TGG Asn Ile Asp His Ser Gly Gly Tyr Val Ala Gln Phe Asn Ile Ser Trp 465 470 475 480	1440
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45	TGC ACT CGT TTA GCT TGG GAA TGG TGG AGA ACG GTA ATT GAT GAC CGG Cys Thr Gly Leu Ala Trp Glu Trp Trp Arg Thr Val Ile Asp Asp Arg 530 535 540	1632
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## (2) INFORMATION FOR SEQ ID NO: 2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 626 amino acids
- (B) TYPE: amino acid
- (C) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

10	Met Thr Asp Val Ser Arg Lys Ile Arg Ala Trp Gly Arg Arg Leu Met	5	10	15	
15	Ile Gly Thr Ala Ala Ala Val Val Leu Pro Gly Leu Val Gly Leu Ala	20	25	30	
20	Gly Gly Ala Ala Thr Ala Gly Ala Phe Ser Arg Pro Gly Leu Pro Val	35	40	45	
25	Glu Tyr Leu Gln Ser Ala Lys Gln Ser Ala Ala Asn Lys Leu His Ser	50	55	60	
30	Ala Gly Gln Ser Thr Lys Asp Ala Ser Ala Phe Asn Lys Glu Asn Ser	65	70	75	80
35	Ile Ser Ser Met Ala Pro Pro Ala Ser Pro Pro Ala Ser Pro Lys Thr	85	90	95	
40	Pro Ile Glu Lys Lys His Ala Asp Glu Ile Asp Lys Tyr Ile Gln Gly	100	105	110	
45	Leu Asp Tyr Asn Lys Asn Asn Val Leu Val Tyr His Gly Asp Ala Val	115	120	125	
50	Thr Asn Val Pro Pro Arg Lys Gly Tyr Lys Asp Gly Asn Glu Tyr Ile	130	135	140	
55	Val Val Glu Lys Lys Lys Ser Ile Asn Gln Asn Asn Ala Asp Ile	145	150	155	160
60	Gln Val Val Asn Ala Ile Ser Ser Leu Thr Tyr Pro Gly Ala Leu Val	165	170	175	
65	Lys Ala Asn Ser Glu Leu Val Glu Asn Gln Pro Asp Val Leu Pro Val	180	185	190	
70	Lys Arg Asp Ser Leu Thr Leu Ser Ile Asp Leu Pro Gly Met Thr Asn	195	200	205	
75	Gln Asp Asn Lys Ile Val Val Lys Asn Ala Thr Lys Ser Asn Val Asn	210	215	220	
80	Asn Ala Val Asn Thr Leu Val Glu Arg Trp Asn Glu Lys Tyr Ala Gln	225	230	235	240
85	Ala Tyr Pro Asn Val Ser Ala Lys Ile Asp Tyr Asp Asp Glu Met Ala	245	250	255	
90	Tyr Ser Glu Ser Gln Leu Ile Ala Lys Phe Gly Thr Ala Phe Lys Ala	260	265	270	
95	Val Asn Asn Ser Leu Asn Val Asn Phe Gly Ala Ile Ser Glu Gly Lys	275	280	285	
100	Met Gln Glu Glu Val Ile Ser Phe Lys Gln Ile Tyr Tyr Asn Val Asn	290	295	300	
105	Val Asn Glu Pro Thr Arg Pro Ser Arg Phe Phe Gly Lys Ala Val Thr	305	310	315	320
110	Lys Glu Gln Leu Gln Ala Leu Gly Val Asn Ala Glu Asn Pro Pro Ala	325	330	335	

Tyr Ile Ser Ser Val Ala Tyr Gly Arg Gln Val Tyr Leu Lys Leu Ser  
 340 345 350  
 Thr Asn Ser His Ser Thr Lys Val Lys Ala Ala Phe Asp Ala Ala Val  
 5 355 360 365  
 Ser Gly Lys Ser Val Ser Gly Asp Val Glu Leu Thr Asn Ile Ile Lys  
 370 375 380  
 10 Asn Ser Ser Phe Lys Ala Val Ile Tyr Gly Gly Ser Ala Lys Asp Glu  
 385 390 395 400  
 Val Gln Ile Ile Asp Gly Asn Leu Gly Asp Leu Arg Asp Ile Leu Lys  
 405 410 415  
 15 Lys Gly Ala Thr Phe Asn Arg Glu Thr Pro Gly Val Pro Ile Ala Tyr  
 420 425 430  
 Thr Thr Asn Phe Leu Lys Asp Asn Glu Leu Ala Val Ile Lys Asn Asn  
 20 435 440 445  
 Ser Glu Tyr Ile Glu Thr Thr Ser Lys Ala Tyr Thr Asp Gly Lys Ile  
 450 455 460  
 25 Asn Ile Asp His Ser Gly Gly Tyr Val Ala Gln Phe Asn Ile Ser Trp  
 465 470 475 480  
 Asp Glu Val Asn Tyr Asp Pro Glu Gly Asn Glu Ile Val Gln His Lys  
 485 490 495  
 30 Asn Trp Ser Glu Asn Asn Lys Ser Lys Leu Ala His Phe Thr Ser Ser  
 500 505 510  
 Ile Tyr Leu Pro Gly Asn Ala Arg Asn Ile Asn Val Tyr Ala Lys Glu  
 515 520 525  
 Cys Thr Gly Leu Ala Trp Glu Trp Trp Arg Thr Val Ile Asp Asp Arg  
 530 535 540  
 40 Asn Leu Pro Leu Val Lys Asn Arg Asn Ile Ser Ile Trp Gly Thr Thr  
 545 550 555 560  
 Leu Tyr Pro Lys Tyr Ser Asn Lys Val Asp Asn Pro Ile Glu Tyr Ala  
 565 570 575  
 45 Leu Ala Tyr Gly Ser Gln Gly Asp Leu Asn Pro Leu Ile Asn Glu Ile  
 580 585 590  
 Ser Lys Ile Ile Ser Ala Ala Val Leu Ser Ser Leu Thr Ser Lys Leu  
 595 600 605  
 50 Pro Ala Glu Phe Val Arg Arg Gly Ser Gly Ile Arg Ser Leu Ser Met  
 610 615 620  
 55 Ser Thr  
 625

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